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(54) Title: ANTISENSE OLIGONUCLEOTIDES AND THERAPEUTIC USE THEREOF IN HUMAN IMMUNODEFICIENCY VIRUS INFECTION			
(57) Abstract  The present invention is directed to oligonucleotides comprising nucleotide sequences sufficiently complementary to conserved regions of human immunodeficiency virus genetic material such that when bound to said region, the oligonucleotides effectively reduce expression of HIV genetic material.			

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ANTISENSE OLIGONUCLEOTIDES AND THERAPEUTIC USE THEREOF  
IN HUMAN IMMUNODEFICIENCY VIRUS INFECTION

Field of Invention

This invention relates to oligonucleotide (ODN)  
5 based therapeutics, particularly the treatment of  
infections of the human immunodeficiency virus (HIV).

Background of the Invention

The present invention relates to ODNs suitable for  
use in treatment of HIV infected individuals by  
10 inhibition of replication of HIV in infected cells.

All references cited herein are incorporated herein  
by reference in their entirety.

HIV is responsible for the disease that has come to  
be known as acquired immune deficiency syndrome (AIDS).  
15 Although initially recognized in 1981, no cure has yet  
been found for this inevitably fatal disease. HIV is  
spread by a variety of means such as sexual contact,  
infected blood or blood products and perinatally.  
Because of the complexity of HIV infection and the  
20 paucity of effective therapies, a great deal of effort  
has been expended in developing methods for detecting,  
treating and preventing infection. Diagnostic procedures  
have been developed for identifying infected persons,  
blood and other biological products.

25 The HIV genome has been well characterized. Its  
approximately 10 kb encode sequences containing  
regulatory segments for HIV replication as well as the  
gag, pol and env genes coding for the core proteins, the  
reverse transcriptase-protease-endonuclease, and the  
30 internal and external envelope glycoproteins,  
respectively. HIV tends to mutate at a high rate causing  
great genetic variation between strains of the viruses  
and indeed between virus particles of a single infected

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individual. There are a few "conserved" regions of the HIV genome which tend not to mutate. Some of these regions are presumed to encode portions of proteins essential for virus function which can thus withstand  
5 very few mutational events.

The HIV env gene encodes the glycoprotein, gp160, which is normally processed by proteolytic cleavage to form gp120, the external viral glycoprotein, and gp41, the viral transmembrane glycoprotein. The gp120 remains  
10 associated with HIV virions by virtue of noncovalent interactions with gp41. These noncovalent interactions are weak, consequently most of the gp120 is released from cells and virions in a soluble form.

Like most viruses, HIV often elicits the production  
15 of neutralizing antibodies. Unlike many other viruses and other infectious agents for which infection leads to protective immunity, however, HIV specific antibodies are insufficient to halt the progression of the disease. Therefore, in the case of HIV, a vaccine that elicits the  
20 immunity of natural infection could prove to be ineffective. In fact, vaccines prepared from the HIV protein gp160 appear to provide little immunity to HIV infection although they elicit neutralizing antibodies. The failure to produce an effective anti-HIV vaccine has  
25 led to the prediction that an effective vaccine will not be available until the end of the 1990's. Therapeutic agents currently used in treatment of AIDS often cause severe side-effects which preclude their use in many patients. It would, thus, be useful to have alternative  
30 methods of treating and preventing the disease that do not entail vaccination and currently available pharmaceutical agents.

Recently, attempts have been made to moderate protein production associated with viral infections by  
35 interfering with the mRNA molecules that direct their synthesis. By interfering with the production of

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proteins, it has been hoped to effect therapeutic results with maximum effect and minimal side effects. It is the general object of such a therapeutic approach to interfere with or otherwise modulate gene expression leading to undesired protein formation.

One method for inhibiting specific gene expression which is believed to have promise is the "antisense" approach. Single-stranded nucleic acid, primarily RNA, is the target molecule for ODNs that are used to inhibit gene expression by an antisense mechanism. The antisense concept and therapeutic approach has been shown to be efficacious. Stein and Cohen (1988) Cancer Res., 48:2659-2668; Walder (1988) Genes & Development, 2:502-504; Marcu-Sekura (1988) Anal. Biochem., 172:289-295; Zon (1987) J. Pro. Chem., 6:131-145; Zon (1988) Pharm. Res., 5:539-549; Van der Krol et al. (1988) Biotechniques, 6:958-973; Loose-Mitchell (1988) TIPS, 2:45-47; Sterling (1992) GEN 12:1; Drug and Development, Inc. (1993) 4:179-195; Ratajczak et al. (1992) Proc. Natl. Acad. Sci. USA 89:11823-11827; and Wagner et al. (1993) Science 260:1510-1513. Antisense ODNs are postulated to exert an effect on target gene expression by hybridizing with a complementary RNA sequence. The hybrid RNA-ODN duplex appears to interfere with one or more aspects of RNA metabolism including processing, translation and metabolic turnover. Chemically modified ODNs have been used to enhance nuclease stability and cell permeability.

Duplex DNA can be specifically recognized by oligomers based on a recognizable nucleomonomer sequence. The motif termed "GT" recognition has been described by Beal et al. (1992) Science, 251:1360-1363; Cooney et al. (1988) Science, 241:456-459; and Hogan et al., EP Publication 375408. In the G-T motif, the ODN is oriented antiparallel to the target purine-rich sequence and A-T pairs are recognized by adenine or thymine residues and G-C pairs by guanine residues.

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Sequence-specific targeting of both single-stranded and duplex target sequences has applications in diagnosis, analysis, and therapy. Under some circumstances wherein such binding is to be effected, it is advantageous to stabilize the resulting duplex or triplex over long time periods.

Covalent crosslinking of the oligomer to the target provides one approach to prolong stabilization. Sequence-specific recognition of single-stranded DNA accompanied by covalent crosslinking has been reported by several groups. For example, Vlassov et al. (1986) Nuc. Acids Res., 14:4065-4076, described covalent bonding of a single-stranded DNA fragment with alkylating derivatives of nucleomonomers complementary to target sequences. A report of similar work by the same group is that by Knorre et al. (1985) Biochimie, 67:785-789. It has also been shown that sequence-specific cleavage of single-stranded DNA can be mediated by incorporation of a modified nucleomonomer which is capable of activating cleavage. Iverson and Dervan (1987) J. Am. Chem. Soc., 109:1241-1243. Covalent crosslinking to a target nucleomonomer has also been effected using an alkylating agent complementary to the single-stranded target nucleomonomer sequence. Meyer et al. (1989) J. Am. Chem. Soc., 111:8517-8519. Photoactivated crosslinking to single-stranded ODNs mediated by psoralen has been disclosed. Lee et al. (1988) Biochem., 27:3197-3203. Use of crosslinking in triple-helix forming probes has also been disclosed. Horne et al. (1990) J. Am. Chem. Soc., 112:2435-2437.

Use of N<sup>4</sup>,N<sup>4</sup>-ethanocytosine as an alkylating agent to crosslink to single-stranded and double-stranded oligomers has also been described. Webb and Matteucci (1986) J. Am. Chem. Soc., 108:2764-2765; (1986) Nuc. Acids Res., 14:7661-7674; and Shaw et al. (1991) J. Am. Chem. Soc., 113:7765-7766. These papers also describe

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the synthesis of ODNs containing derivatized cytosine. The synthesis of oligomers containing N<sup>6</sup>,N<sup>6</sup>-ethanoadenine and the crosslinking properties of this residue in the context of an ODN binding to a single-stranded DNA has  
5 been described. Matteucci and Webb (1987) Tetrahedron Letters, 28:2469-2472.

In a recent paper, sequence-specific binding of an octathymidylate conjugated to a photoactivatable crosslinking agent to both single-stranded and double-  
10 stranded DNA is described. Praseuth et al. (1988) Proc. Natl. Acad. Sci. (USA), 85:1349-1353. In addition, targeting duplex DNA with an alkylating agent linked through a 5'-phosphate of an ODN has been described. Vlassov et al. (1988) Gene 313-322; and Fedorova et al.  
15 (1988) FEBS Lett., 228:273-276.

In effecting binding to obtain a triplex, to provide for instances wherein purine residues are concentrated on one chain of the target and then on the opposite chain, oligomers of inverted polarity can be provided. By  
20 "inverted polarity" is meant that the oligomer contains tandem sequences which have opposite polarity, i.e., one having polarity 5'→3' followed by another with polarity 3'→5', or vice versa. This implies that these sequences are joined by linkages which can be thought of as  
25 effectively a 3'-3' internucleoside junction (however the linkage is accomplished), or effectively a 5'-5' internucleoside junction. Such oligomers have been suggested as by-products of reactions to obtain cyclic ODNs. Capobionco et al. (1990) Nuc. Acids Res., 18:2661-  
30 2669. Compositions of "parallel-stranded DNA" designed to form hairpins secured with AT linkages using either a 3'-3' inversion or a 5'-5' inversion have been synthesized. van de Sande et al. (1988) Science, 241:551-557. In addition, triple helix formation using  
35 oligomers which contain 3'-3' linkages have been described. Horne and Dervan (1990) J. Am. Chem. Soc.,

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112:2435-2437; and Froehler et al. (1992) Biochem.,  
31:1603-1609.

The use of triple helix (or triplex) complexes as a means for inhibition of the expression of target gene expression has been previously adduced (International Application No. PCT/US89/05769). Triple helix structures have been shown to interfere with target gene expression, demonstrating the feasibility of this approach. International Application No. PCT/US91/09321; and Young et al. (1991) Proc. Natl. Acad. Sci. USA, 88:10023-10026.

Various modifications have been found to be suitable for use in ODNs. Oligomers containing 5-propynyl modified pyrimidines have been described. Froehler et al. (1992) Tetrahedron Letters, 33:5307-5310. 2'-Deoxy-7-deazaadenosine and 2'-deoxy-7-deazaguanosine have been incorporated into ODNs and assessed for binding to the complementary DNA sequences. Thermal denaturation analysis (T<sub>m</sub>) has shown that these substitutions modestly decrease the T<sub>m</sub> of the duplex when these analogs are substituted for 2'-deoxyadenosine and 2'-deoxyguanosine. Seela and Kehne (1987) Biochem., 26:2232-2238; and Seela and Driller (1986) Nuc. Acids Res., 14:2319-2332. It has also been shown that ODNs which alternate 2'-deoxy-7-deaza-adenosine and -thymidine can have a slightly enhanced duplex T<sub>m</sub> over ODNs containing 2'-deoxy-adenosine and -thymidine. Seela and Kehne (1985) Biochem., 24:7556-7561.

DNA synthesis via amidite and hydrogen phosphonate chemistries has been described. U.S. Patent Nos. 4,725,677; 4,415,732; 4,458,066; and 4,959,463.

The use of antisense ODNs in treatment of HIV has been described by several investigators. Matthews and Kricka (1988) Anal. Biochem. 169:1-25; Degols et al. (1992) Antiviral Res. 279-287; and U.S. Patent No. 5,110,802 to Cantin et al. Prior attempts at antisense targeting to HIV have been focused on inhibition of the



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synthesis of viral proteins essential to the success of the infection by using de novo infection of susceptible cells in culture. Agrawal et al. (1988) Proc. Natl. Acad. Sci. USA 85:7079-7083; Goodchild et al. (1988) Proc. Natl. Acad. Sci. USA 85:5507-5511; and Zamecnik et al. (1986) Proc. Natl. Acad. Sci. USA 83:4143-4146. By its very nature, such an assay allows a test compound to potentially interfere with any critical processes within the virus life cycle. Consequently, de novo infection assays are efficient for initial screening of compounds for antiviral activity but are complex systems for establishing mechanism(s) of actions, including whether the antiviral activity exhibited by an intended antisense oligomer actually derives from an antisense process or is the result of an action operative at an early stage of the infection process.

Infection of T lymphocytes with HIV results in a cytopathic effect and cell death that has been linked to a selective loss of the helper T-lymphocyte function of the immune system. Arya et al. (1985) Science 29:69-73. In addition to acute infection, which leads to cell death through the accumulation of HIV-1 antigens within the infected cell (Petteway et al. (1991) TIPS 12:28-34) a chronic or persistent infection also occurs. The persistence of these viral reservoirs has been implicated in the progression of HIV infection and AIDS. Noe et al. (1988) Biochim. Biophys. Acta 946:253-260. Rational drug discovery targeted to late-stage events in HIV replication has the potential to yield antiviral agents capable of blocking virus spread by inhibiting the production of infectious virions from this chronic reservoir.

It has now been found that inhibition of viral gene expression and replication can be more efficiently achieved by targeting the conserved sites of the viral

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RNAs that signal the synthesis of conserved HIV proteins, particularly the p24 core antigen protein.

### Summary of the Invention

The present invention is directed to ODNs comprising  
5 nucleotide sequences sufficiently complementary to conserved regions of human immunodeficiency virus (HIV) genetic material such that when bound to said region, the ODNs effectively reduce expression of the HIV genetic material.

### Brief Description of the Drawings

10 Figure 1 is an autoradiograph of a SDS-PAGE showing *in vivo* synthesis of HIV proteins and their breakdown products.

Figure 2 is an autoradiograph of a SDS-PAGE showing  
15 significant inhibition of expression of HIV proteins by antisense ODN directed against the first splice donor site of the HIV-1 genome.

Figure 3 is a Western blot analysis showing the effects of different concentrations of antisense ODNs  
20 directed against the first donor site of the HIV genome directed against HIV-gene product synthesis.

Figure 4 is an autoradiograph of a SDS-PAGE showing the concentration dependent inhibitory effects of antisense ODN, GPI2A, on HIV-1 viral gene expression in a  
25 COS-like monkey kidney cell line stably transfected with pGAGPOL-rre-r (HIV-1 gene sequence), called B4-14.

Figure 5 depicts the potentiation of the cell-associated radiolabeled ODN through formulation with lipofectin.

30 Figure 6 depicts the inhibitory activity of 1  $\mu$ M GPI2A on the biosynthesis of HIV-1 gag protein precursor p55.

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Figure 7 depicts the sequence-specific inhibitory activity of 1  $\mu$ M GPI2A on the biosynthesis of HIV-1 viral core antigen p24.

Figure 8 depicts the densitometry analysis of an  
5 autoradiograph showing concentration-dependent inhibitory activity of GPI2A on the biosynthesis of HIV gag protein p24.

Figure 9 depicts the inhibitory activity of 1  $\mu$ M GPI2A on HIV-1 mRNA. A: Total RNA extraction after 4  
10 hour pretreatment with ODN; B: After 24 hour pretreatment.

Figure 10 (concentration-dependent inhibition of RT-activity by GPI2A in virus infected P-PHA stimulated CBMC cells) depicts the inhibition of HIV-1 replication by  
15 GPI2A in acutely infected cells.

Figure 11 depicts the inhibition of HIV-1 replication in chronically infected H9/IIIB cells.

Figure 12 depicts the colony-forming ability of pretreated and untreated B4.14 cells.

20 Figure 13 depicts the effects of ODN pretreatment on the growth characteristics of B4.14 cells as compared to untreated controls.

#### Description of the Invention

Several regions within HIV RNA have now been found  
25 to be effective targets for the inhibition of expression of viral gene products by antisense ODNs and their analogues. The inhibition is based on the capacity to block certain functions during viral replication as measured by production of p24. The clinical importance  
30 of p24, a cleavage product of p55, is evidenced by the fact that serum levels of antibody to p24 antigen of HIV provide evidence of the effectiveness of immune response to the virus as well as serving as a marker of free virus in the serum of patients with advanced stage AIDS.  
35 Goedert et al. (1989) N. Engl. J. Med., 321:114.

According to the present invention, two 20mer ODN sequences were designed and tested for their efficacy as

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anti-HIV chemotherapeutic agents. While not being bound by any one theory, the mechanism of action of antisense chemotherapeutics may be solely due to binding to the mRNA or DNA so as to prevent translation or

5 transcription, respectively; the mechanism of action may also be due to activation of RNase H and subsequent degradation of the RNA.

The sequences are conserved in at least two different HIV isolates (Battles et al. (1992) J. Virol.  
10 66:6868), and, therefore the antisense ODNs may be effective agents against a wide variety of HIV strains.

The sequences were synthesized based on the phosphoramidite chemistry of ODN synthesis on Applied Biosystems model 380D automated DNA and 392 DNA/RNA  
15 synthesizer. They were purified using ODN purification cartridges, PAGE and/or HPLC.

In therapeutic applications, the ODNs are utilized in a manner appropriate for treatment of a variety of conditions by inhibiting expression of the target genetic  
20 regions. For such therapy, the ODNs, alone or in combination can be formulated for a variety of modes of administration, including systemic, topical or localized administration. Techniques and formulations generally can be found in Remington's Pharmaceutical Sciences, Mack  
25 Publishing Co., Easton, PA, latest edition. The ODN active ingredient is generally combined with a pharmaceutically acceptable carrier such as a diluent or excipient which can include fillers, extenders, binders, wetting agents, disintegrants, surface-active agents, or  
30 lubricants, depending on the nature of the mode of administration and dosage forms. Typical dosage forms include tablets, powders, liquid preparations including suspensions, emulsions and solutions, granules, capsules and suppositories, as well as liquid preparations for  
35 injections, including liposome preparations.

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Preferably, the compositions include an effective concentration of a lipid formulation. An effective concentration is one which enhances uptake of the ODNs by cells. It is within the skill of one in the art to  
5 determine whether a particular lipid formulation or concentration thereof is effective in enhancing uptake and examples are provided below. Suitable lipid formulations include, but are not limited to, lipid mixtures, liposomes and cationic lipid preparations. A  
10 suitable cationic lipid formulation is lipofectin.

For systemic administration, injection is preferred, including intramuscular, intravenous, intraperitoneal, and subcutaneous. For injection, the ODNs of the invention are formulated in liquid solutions, preferably  
15 in physiologically compatible buffers such as Hank's solution or Ringer's solution. In addition, the ODNs can be formulated in solid form and redissolved or suspended immediately prior to use. Lyophilized forms are also included. Dosages that can be used for systemic  
20 administration preferably range from about 0.01 mg/Kg to 50 mg/Kg administered once or twice per day. However, different dosing schedules can be utilized depending on (i) the potency of an individual ODN at inhibiting the activity of its target DNA or RNA, (ii) the severity or  
25 extent of the pathological disease state, or (iii) the pharmacokinetic behavior of a given ODN.

Systemic administration can also be by transmucosal or transdermal means, or the compounds can be administered orally. For transmucosal or transdermal  
30 administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, bile salts and fusidic acid derivatives for transmucosal administration. In addition, enhancers can  
35 be used to facilitate permeation. Transmucosal administration can be through use of nasal sprays, for example,

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or suppositories. For oral administration, the ODNs are formulated into conventional oral administration forms such as capsules, tablets, and tonics.

For topical administration, the ODNs of the  
5 invention are formulated into ointments, salves, gels, or creams, as is generally known in the art.

Formulation of the invention oligomers for ocular indications is based on standard compositions known in the art.

10 In addition to use in therapy, the ODNs of the invention can be used as diagnostic reagents to detect the presence or absence of the target nucleic acid sequences to which they specifically bind. The enhanced  
binding affinity of the invention ODNs is an advantage  
15 for their use as primers and probes. Diagnostic tests can be conducted by hybridization through either double or triple helix formation which is then detected by  
conventional means. For example, the ODNs can be labeled  
using radioactive, fluorescent, or chromogenic labels and  
20 the presence of label bound to solid support detected. Alternatively, the presence of a double or triple helix can be detected by antibodies which specifically recognize these forms.

The use of ODNs containing the invention substitute  
25 linkages as diagnostic agents by triple helix formation is advantageous since triple helices form under mild conditions and the assays can thus be carried out without subjecting test specimens to harsh conditions.  
Diagnostic assays based on detection of RNA often require  
30 isolation of RNA from samples or organisms grown in the laboratory, which is laborious and time consuming, as RNA is extremely sensitive to ubiquitous nucleases.

The ODN probes can also incorporate additional  
modifications such as modified sugars and/or substitute  
35 linkages that render the ODN especially nuclease stable, and would thus be useful for assays conducted in the

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presence of cell or tissue extracts which normally contain nuclease activity. ODNs containing terminal modifications often retain their capacity to bind to complementary sequences without loss of specificity.

- 5 Uhlmann et al. (1990) Chem. Rev., 90:543-584. As set forth above, the invention probes can also contain linkers that permit specific binding to alternate DNA strands by incorporating a linker that permits such binding. Froehler et al. (1992) Biochem., 31:1603-1609;  
10 and Horne et al. (1990) J. Am. Chem. Soc., 112:2435-2437.

Incorporation of base analogs into probes that also contain covalent crosslinking agents has the potential to increase sensitivity and reduce background in diagnostic or detection assays. In addition, the use of  
15 crosslinking agents will permit novel assay modifications such as (1) the use of the crosslink to increase probe discrimination, (2) incorporation of a denaturing wash step to reduce background and (3) carrying out hybridization and crosslinking at or near the melting  
20 temperature of the hybrid to reduce secondary structure in the target and to increase probe specificity. Modifications of hybridization conditions have been previously described. Gamper et al. (1986) Nuc. Acids Res., 14:9943.

- 25 ODNs of the invention are suitable for use in diagnostic assays that employ methods wherein either the oligomer or nucleic acid to be detected are covalently attached to a solid support as described in U.S. Patent No. 4,775,619. The ODNs are also suitable for use in  
30 diagnostic assays that rely on polymerase chain reaction (PCR) techniques to amplify target sequences according to methods described, for instance, in European Patent Publication No. 0 393 744. ODNs of the invention containing a 3' terminus that can serve as a primer are  
35 compatible with polymerases used in PCR methods such as the Tag or Vent<sup>TM</sup> (New England Biolabs) polymerase. ODNs

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of the invention can thus be utilized as primers in PCR protocols.

The ODNs are useful as primers that are discrete sequences or as primers with a random sequence. Random  
5 sequence primers can be generally about 6, 7, or 8 nucleomonomers in length. Such primers can be used in various nucleic acid amplification protocols (PCR, ligase chain reaction, etc.) or in cloning protocols. The substitute linkages of the invention generally do not  
10 interfere with the capacity of the ODN to function as a primer. ODNs of the invention having 2'-modifications at sites other than the 3' terminal residue, other modifications that render the ODN RNase incompetent or otherwise nuclease stable can be advantageously used as  
15 probes or primers for RNA or DNA sequences in cellular extracts or other solutions that contain nucleases. Thus, the ODNs can be used in protocols for amplifying nucleic acid in a sample by mixing the ODN with a sample containing target nucleic acid, followed by hybridization  
20 of the ODN with the target nucleic acid and amplifying the target nucleic acid by PCR, LCR or other suitable methods.

The ODNs derivatized to chelating agents such as EDTA, DTPA or analogs of 1,2-diaminocyclohexane acetic  
25 acid can be utilized in various in vitro diagnostic assays as described in, for instance, U.S. Patent Nos. 4,772,548, 4,707,440 and 4,707,352. Alternatively, ODNs of the invention can be derivatized with crosslinking agents such as 5-(3-iodoacetamidoprop-1-yl)-2'-  
30 deoxyuridine or 5-(3-(4-bromobutyramido)prop-1-yl)-2'-deoxyuridine and used in various assay methods or kits as described in, for instance, International Publication No. WO 90/14353.

In addition to the foregoing uses, the ability of  
35 the oligomers to inhibit gene expression can be verified in in vitro systems by measuring the levels of expression



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in subject cells or in recombinant systems, by any suitable method. Graessmann et al. (1991) Nuc. Acids Res., 19:53-59. In the present case, levels of p24 have been measured as indicative of virus replication.

5       The first embodiment of the present invention is an ODN complementary to the region between the 5' long terminal repeat (LTR) and the first initiation codon (AUG) of the gag gene. This region contains highly conserved sequences required for efficient viral RNA  
10 packaging. Klotman and Wong-Staal (1991) in: The Human Retroviruses by Gallo & Jay, eds. Acad. Press. The antisense ODN is referred to as "anti-gag." The ODN is of sufficient length and complementarity to inhibit HIV-1 gene expression. The complementary site is from bases  
15 +262 to +281 as numbered according to Ratner et al. (1985) Nature, 313:277-283. In a preferred embodiment the anti-gag ODN has the specific sequence:

5' CCGCCCCTCGCCTCTTGCCG 3'

20       The second embodiment of the present invention is an ODN complementary to the region within the second splice acceptor site. (Battles et al., 1992.) The antisense ODN is referred to as "GPI2A." The ODN is of sufficient length and complementarity to inhibit HIV-1 gene expression. The complementary site is from bases +1189  
25 to +1208 as numbered according to Ratner et al. (1985). In a preferred embodiment the GPI2A ODN has the specific sequence:

5' GGTTCCTTTTGGTCCTTGCTCT 3'

30       In a further preferred embodiment, the ODNs were chemically modified by substitution of at least one of the naturally occurring non-bridging oxygen atoms of the phosphodiester backbone with a sulfur to form the corresponding phosphorothioate derivatives of the oligomers. The preferred positions of the sulfur atoms  
35 are as shown below.

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Anti-gag:

5' C'CG'CC'CC'TC'GC'CTC'TTG'CC'G 3'; and

GPI2A:

5' G'GTTC'TTTTG'GTCC'TTG'TC'T 3'.

5 In accordance with the present invention, methods of modulating the expression of the p24 protein are provided. The targeted RNA, or cells containing it, are treated with the ODN analogs which bind to specific regions of the RNA coding for the HIV p24 core structural protein. The RNA targeted sites include regions involved in the mechanism of expression of the HIV p24 core structural protein.

The following examples are intended to illustrate, but not to limit, the invention. The examples show that sequence-specific inhibition of the viral core antigen production in the presence of 1  $\mu$ M GPI2A was also observed. The densitometric analysis (over 84% at 1  $\mu$ M) of the autoradiograph showing the concentration-dependent inhibitory effect of GPI2A on the p24 formation gave an IC<sub>50</sub> of 0.5  $\mu$ M. Northern analysis showed dramatic reduction in the mRNA levels obtained from cells pretreated with GPI2A. The inhibition pattern agrees with the data obtained at the protein level since both effects were observed even after 4 hrs pretreatment. The cytotoxic effects of the ODNs were evaluated by the colony-forming ability and the growth characteristics of the cells.

The concentration-dependent attenuation of the viral protein biosynthesis indicates pharmacological value of GPI2A. The inhibitory effect resulting from lower transcript level points to a possible mechanism of action via RNase H activation, thereby supporting the proposed mechanism of action of the phosphorothioate derivatives of the antisense ODN. This approach will perhaps be of significant contribution to the general screening for the development of antisense oligomers as drug candidates for

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effective cure of HIV infection and most major diseases that could be combated by modulating the expression of functionally relevant disease-associated proteins of therapeutic importance.

5

Example 1Cell Culture

To determine the effect of antisense oligomer on viral gene expression, B4.14 cells, provided by Dr. David Rekosh, Microbiology Department, University of Virginia, were seeded at a cell density of 5,000-12,000 cells per well in 12-well/35 mm plastic tissue culture plates and were maintained in Iscove's Modified Dulbecco's Medium with 10% calf serum, 50 µg/ml gentamycin and 200 µg/ml hygromycin B at 37°C in a humidified incubator with 5% CO<sub>2</sub> for a few hours. Subsequently, the incubated cells were washed and incubated under the same conditions with medium containing the indicated concentrations of oligomer and 10% heat inactivated serum to reduce serum nuclease activity. The same oligomer sequences, but with switched polarity were used as controls.

Example 2Viral Antigen Assay

Cells cultured as described in Example 1 were labeled with 75 to 150 µCi/ml [<sup>35</sup>S]-methionine (70% L-Methionine/15% L-Cysteine) in the presence of methionine-free medium containing 29.2 mg/100 ml glutamine, 50 µg/ml gentamycin, 200 µg/ml hygromycin B, 10% heat inactivated fetal calf serum plus the desired concentration of oligomer. The [<sup>35</sup>S]-methionine concentration was 185 MBq and the specific activity was 1057 Ci/mmol. Labeled samples were subsequently washed with phosphate buffered saline (PBS) and resuspended in 200 µl lysis buffer comprised of 50 mM Tris, pH 7.2; 150 mM NaCl; 5 mM EDTA; 1% Triton-100; 0.2% Deoxycholic acid.

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Culture medium containing labeled virus was treated with 10% Triton X-100 to a 1% final concentration to disrupt virus particles. The samples were preabsorbed with protein A-Sepharose beads for 30 min. at 4°C. [<sup>35</sup>S]-methionine-labeled viral proteins were then immunoprecipitated for 2 hours using protein A-Sepharose beads and 2.5 µl/sample of polyclonal rabbit antiserum directed against HIV-1 p25/24. The antibodies were obtained from National Institute of Allergy & Infectious Disease (AIDS Research & Reference Reagent Program) and MicroGeneSys, Inc.

The resulting pellets were washed 4 times with lysis buffer, once with lysis buffer containing 500 mM NaCl and finally once with TNE buffer comprised of 10 mM Tris, pH 7.2; 25 mM NaCl; 1 mM EDTA. Samples were then resuspended in 20 - 30 µl 2X SDS sample buffer, boiled for 5 - 10 min, applied to a 12.5% SDS polyacrylamide gel electrophoresis and then analyzed by electrophoresis, according to the method described by Laemmli (1970) Nature, 227:680-685. The results obtained are depicted in Figures 1 and 2. Percent inhibition is determined by densitometric analysis of the autoradiography. The ODNs were phosphorothioate derivatives (sulfurization on alternate bases).

The inhibition of viral protein synthesis (CCGCCCCTCGCCTCTTGCCG; complementary site 262-281; function, Splice Donor) by anti-gag in B4.14 cells was 30% (Figure 2).

Figure 1 is an autoradiograph of a SDS-PAGE showing in vivo synthesis of HIV-1 viral proteins and their breakdown products. Two hundred µl each of CMT3 [wild-type (left)] and B4.14 [transfected line (right)] cell lysates following metabolic labeling with [<sup>35</sup>S]-methionine were immunoprecipitated with rabbit serum against p24 viral antigen as described above were run on an SDS-PAGE and exposed to X-ray film. The positions of the viral

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proteins (p160; p55 and p24) are clearly visible in the B4.14 cell lysate but not in control cell line CMT3 cell lysate.

Figure 2 is an autoradiograph of a SDS-PAGE showing a significant inhibition of expression of HIV proteins by the anti-gag ODN. Two hundred  $\mu$ l of B4.14 [transfected cell line] cell lysates following 6 days treatment with antisense [AS]; sense, the inverse complement of the antisense ODN [S]; and control (B4.14, cell lysate only); and subsequent  $^{35}$ S-methionine labeling were immunoprecipitated with rabbit serum directed against p24 viral antigen as described above. Equal amounts of protein were added in each lane.

### Example 3

#### The Effects of Different Concentrations of the Antisense ODNs

To determine whether there was a dose relationship of the antisense ODNs on HIV gene expression, the following experiment was performed.

The cells were cultured as described in Example 1 and incubated overnight with different concentrations of anti-gag ODNs in the presence of 5  $\mu$ g/ml Lipofectin and 1% heat-inactivated FCS. The medium was subsequently replaced with fresh medium containing 10% heat-inactivated serum. ODN was then added and incubated for 7 days. Western blot analysis was performed with rabbit polyclonal antibody directed against HIV p24/55 proteins.

Following SDS-polyacrylamide electrophoresis, cellular proteins were electrophoretically transferred to Immobilon membrane (Schleicher and Schuell) as follows. An Immobilon membrane was placed in methanol in a clean dish, washed several times in deionized distilled water and soaked in western transfer buffer (60.6 g Tris-HCl; 288 g glycine; 4 l methanol and distilled water to 20 l). The apparatus used is the Bio-Rad Trans-Blot cell, used

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according to the manufacturer's instructions. Western blot analysis was performed using the Vectastain ABC kit (Alkaline Phosphatase Rapid IgG) (Vector Laboratories) according to the manufacturer's instructions.

- 5       The results are shown in Figure 3 where it can be seen that 0.5 and 1  $\mu$ M antisense ODN were equally effective at preventing HIV-1 viral protein synthesis.

#### Example 4

##### The Effects of Different

##### Concentrations of the ODN GPI2A

10       To determine the ability of the ODN GPI2A to inhibit expression of p24 in HIV infected cells, the following experiments were performed.

- Cells were incubated overnight with 0.1, 0.5 and 1.0  
15    $\mu$ M of the ODN in the presence of 1% heat-inactivated fetal calf serum. The serum concentration was subsequently raised to 10% and incubated for 3 days. About  $3 \times 10^7$  cpm/probe was immunoprecipitated using rabbit polyclonal antibody directed against p24/25 viral  
20   proteins as described above. The lane marked control had the sense strand, the inverse complement of the antisense oligomer. The autoradiograph in Figure 4 shows that there was a dose-dependent inhibition of the HIV viral core antigen, among others.

25

#### Example 5

##### Construction of the Expression Plasmids

- pCMVrev contains the coding sequence from a HIV-1 cDNA fragment under the control of the promoter-enhancer region from the simian cytomegalovirus (CMV) IE94 gene (-  
30   650 to +30). Jeang et al. (1987) J. Virol. 61:1559-1570. This plasmid was constructed by inserting the Bsu961 fragment from pCV1 into the vector pCMV. In pCMV, the SV40 sequences contained within pBABY have been exchanged

-21-

for the CMV promoter region. Lewis et al. (1990) J. Virol. 64:1690-1697.

pGAGPOL contains a cDNA fragment from the proviral BH10 clone of HIV-1 (corresponding to HXB2 nucleotides 679-5785) inserted into the XhoI site of pBABY. This fragment includes the open reading frames for gag, pol and vif. It was isolated from a plasmid which contained the entire BH10 proviral clone as a SallI fragment in the vector pSP64 (Promega Biotec, Madison, WI). The HIV-1 sequences were excised as a SallI fragment between the SallI site in the pSP64 polylinker and the SallI site at 5785 in the HIV-1 DNA. After inserting into pBABY, a small deletion (AvaI-XbaI) was made in the remaining pSP64 polylinker by restriction enzyme digestion, T4 DNA polymerase repair and religation resulting in deletion of a BamHI site (polylinker). A second BamHI site at the boundary of the SV40 and pBR322 DNA sequences was also removed by restriction enzyme digestion and repair. These manipulations were performed to facilitate the construction of pGAGPOL-RRE-r. pGAGPOL-RRE-r was created by inserting the BglII-BamHI fragment from HIV-1 BH10 clone (corresponding to HXB2 nucleotides 7620 to 8474) into the unique BamHI site present in pGAGPOL in the same orientation with respect to gag, pol and vif as in the viral genome.

#### Example 6

##### Transfected Cells and Their Culture

A COS-like Monkey Kidney cell line, CMT3 (Gerard and Glutzman (1985) Mol. Cell Biol 5:3231-3240) was transfected with pCMVgagpol-rre-r, pCMVrev which is required for efficient expression of gag (Smith et al. (1990) J. Virol. 64:2743-2750) and a selective marker plasmid that expresses the hygromycin resistance gene by a standard CaPO<sub>4</sub> method (Graham and van der Eb (1973) Virology 52:456) to obtain the cell line B4.14 (Dr. David

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Rekosh, Microbiol. Dept., University of Virginia). The cells were maintained in Iscove's Modified Dulbecco's Medium with 10% calf serum, 50 µg/ml gentamycin and 200 µg/ml hygromycin B.

- 5        Umbilical cord blood mononuclear cells (CBMC) were isolated by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) gradient centrifugation. The cells were collected, washed and phytohemagglutinin (P-PHA)-stimulated. The cells were seeded in culture flasks and maintained for  
10 three days in complete RPMI-1640 culture medium (Gibco Laboratories, Toronto, Ontario, Canada) supplemented with 10% heat-inactivated fetal bovine serum (Flow Laboratories, Toronto, Ontario, Canada), 2 mM L-glutamine, and 200 U/ml penicillin. Bour et al. (1991)  
15 J. Virol. 65:6387-6396.

#### Example 7

##### Synthesis of Oligodeoxynucleotide Sequences

- Using a computer model program [OLIGO: Primer Analysis Software, Version 3.4], which calculates the  
20 free energy (delta G), hybridization temperature and secondary structure of DNA and RNA based on the highly accurate measurement of nearest-neighbor delta G values, antisense oligodeoxynucleotide that is highly selective for the intended target sequence was generated. The  
25 oligodeoxynucleotide thus generated was expected to have minimal genetic variability (Battles et al. (1992) J. Virol. 66:6868-6877), to form stable duplexes and non-self-complementary. GPI2A is an antisense construct made of the following base composition according to the  
30 nomenclature of Ratner et al. (1985) Nature 313:277-284: 'GGTTCTTTTGGTCCTTGCTCT', the two point mutations (underlined): 'GGTTCTTTTGTGCCTTGCTCT', and the sense strand: 'TCTGTTCCCTGGTTTCTTGG' spanning nucleotides +1189-+1208, a genetically conserved region in many  
35 retroviruses. Battles et al. (1992); Garvey et al.



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- (1990) Virology 175:391-409; and Gonda et al. (1987) Nature 330:388-391. Within this stretch, BIV shared 8 of 10 residues with EIAV, 6 of 10 with SIV<sub>mac</sub>, 3 of 10 with visna virus, and 7 of 10 with SIV<sub>gsm</sub>, HIV-2 and HIV-1.
- 5 Garvey et al. (1990). GPI2A was chemically modified at 7 different base positions by substitution of the naturally occurring nonbridging oxygen atoms of the phosphodiester backbone with sulfur atoms to form the corresponding relatively nuclease-resistant phosphorothioate
- 10 derivative. Cohen (1989) Trends Pharmacological Sci. 10:435-437; and Zon (1988) Pharmaceut. Res. 5:539-548.

ODNs were synthesized on Applied Biosystems models 380B automated DNA and 392 DNA/RNA synthesizers using standard phosphoramidate chemistry. The oligomers and

15 their phosphorothioate analogs were purified using oligonucleotide purification cartridges (Applied Biosystem), 20% denaturing polyacrylamide gel electrophoresis, PAGE (Efcaitch (1990) in D. Rickwood and B.D. Hames (eds.), Gel Electrophoresis of Nucleic

20 Acids - A Practical Approach Oxford University Press. Oxford) or by high performance liquid chromatography, HPLC (Zon and Thompson. (1986) BioChromatog. 1:22-32).

#### Example 8

##### Pretreatment of Cells with

##### ODN/Lipid Formulation

25 Previous studies indicated modest antisense activity of GPI2A due to poor cell membrane permeability. To optimize this effect, a cationic lipid delivery system was employed to offset the high cost of ODN synthesis.

30 Lipofectin reagent is a 1:1 [w/w] liposome formulation of N-[1-(2,3-dioleoyloxy)propyl-N,N,N-trimethylammonium chloride (DOTMA), and dioleoyl phosphatidylethanolamine (DOPE) in membrane-filtered water (GIBCO BRL, Research Products Life Sci. Inc.).

35 Lipofectin reagent reacts spontaneously with DNA to form

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a lipid-DNA complex with complete entrapment of the DNA. The fusion of the complex with the cell membrane results in efficient uptake of the encapsulated DNA into the intracellular environment of the cell. Juliano and

- 5 Akhtar (1992) Antisense Res. & Develop. 2:165-176.

Appropriate amounts of DNA and lipofectin reagent were diluted separately in sterile distilled water and were subsequently combined in a polystyrene tube and mixed gently without vortexing. The mixture was then  
10 allowed to stand for at least 15 min at room temperature. Felgner et al. (1987) Proc. Natl. Acad. Sci. USA 84:7413-7417. About 50-70% confluent plates were washed with serum-free medium. One ml of medium was then added to the cells and the complex added gently dropwise, as  
15 uniformly as possible. Depending on the experimental conditions, cells were incubated 0.5 min to 24 h at 37°C in a humidified 5-10% carbon dioxide environment. The cells were either labeled for immunoprecipitation or the medium and ODN subsequently replaced and the cells  
20 incubated for the desired length of time in the presence of ODN prior to radiolabeling and immunoprecipitation by a modification of a previously published procedure. Chan et al. (1993) Biochem. 47:12835-12840.

To measure the cell-associated radioactivity, cells  
25 were incubated with <sup>32</sup>P-labeled 5'-DNA terminus labeling system (GIBCO, BRL). ODNs in the presence or absence of lipofectin reagent and the cell-associated radioactivity were determined by direct suspension of the labeled cells in liquid scintillation medium (Universol, ICN  
30 Biomedicals Inc. Irvine, CA). Cells were seeded at a density of  $1.5 \times 10^4$  and were subsequently incubated at 37°C in a humidified 5-10% carbon dioxide environment in the presence of 1  $\mu$ M radiolabeled GPI2A with or without 10  $\mu$ g/ml lipofectin reagent for 1 and 4 h. The cells  
35 were washed with PBS and the cell-associated radioactivity (cpm, y-axis) was counted by lysis and

-25-

suspension in liquid scintillation medium. The results obtained are presented in Figure 5. In Figure 5, each point represents an average value of four experiments. Statistical analysis was performed by student's t-Test:

- 5 For -Lipo(1h) vs. +Lipo(1h)  $p < 0.01$ ; -Lipo(4h) vs. +Lipo(4h)  $p < 0.005$ ; -Lipo(1h) vs. -Lipo(4h)  $p < 0.01$ ; +Lipo(1h) vs. +Lipo(4h)  $p < 0.003$ .

#### Example 9

##### Antisense Oligomer and Viral Gene Expression

- 10 To determine the effect of antisense oligomer on viral gene expression, B4.14 cells were seeded at the desired cell density into 6-well plastic tissue culture plates and were maintained in Iscove's Modified Dulbecco's Medium with 10% calf serum, 50  $\mu\text{g/ml}$
- 15 gentamycin and 200  $\mu\text{g/ml}$  hygromycin B at 37°C in a humidified incubator with 5%  $\text{CO}_2$ . Subsequently, cells were washed and incubated four 4 hr with medium containing 1  $\mu\text{M}$  GPI2A and heat-inactivated serum. The control oligomer sequences contained two point mutations
- 20 or were the inverse complement of the antisense construct.

- The pretreated cells and the controls were then washed with PBS and radiolabeled for 30 min with 120-250  $\mu\text{Ci/ml}$   $^{35}\text{S}$ -Methionine (70% L-Methionine/15% L-Cysteine.
- 25 [ $^{35}\text{S}$ ]-Methionine concentration: 185 MBq. Specific Activity: 1057 Ci/mmol) in the presence of methionine-free medium. Labeled samples were subsequently washed with PBS buffer and resuspended in 200  $\mu\text{l}$  lysis buffer (50 mM Tris pH 7.2, 150 mM NaCl, 5 mM EDTA, 1% Triton X-
- 30 100, 0.2% deoxycholic acid).

- The samples were precleared with protein A-sepharose beads for at least 30 min. at 4°C. [ $^{35}\text{S}$ ]-methionine-labeled viral proteins were immunoprecipitated for 2 hours or overnight at 4°C using protein A-sepharose beads
- 35 and 2.5  $\mu\text{l}$  sample of polyclonal rabbit antiserum directed

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against HIV-1 p25/24 (National Institute of Allergy & Infectious Diseases [AIDS Research & Reference Reagent Program) and MicroGeneSys, Inc.) The resulting pellets were washed 4 times with lysis buffer, once with lysis  
5 buffer containing 500 mM NaCl and finally once with TNE buffer (10 mM Tris pH 7.2; 25 mM NaCl; 1 mM EDTA). Samples were then resuspended in 20-30  $\mu$ l 2x SDS sample buffer, boiled for 5-10 min. and were analyzed on a 12.5% SDS-PAGE dried and exposed on a KODAK X-OMAT film  
10 (Eastman Kodak, Rochester, NY). The results obtained are presented in Figures 6-8.

#### Example 10

##### Northern Analysis

Northern analyses on the isolated RNA samples were  
15 by a modification of a previously published procedure. Amara et al. (1993) Nuc. Acids Res. 21:4803-4809. Briefly, cells were pretreated with 1  $\mu$ M of ODN in the presence of 10  $\mu$ g/ml lipofectin reagent as described in Example 8. Messenger RNAs were extracted from the cells  
20 by the rapid RNA preparation method according to the method described by Gough (1988) Analytical Biochemistry 173:93-95. Electrophoresis was performed on 1% formaldehyde agarose gels followed by blotting onto nylon membranes (Schleicher & Schuell, Nytran<sup>+</sup>, 0.45  $\mu$ m)  
25 overnight, dried and subsequently baked at 80°C for 2 hrs. The filters were prehybridized at 42°C overnight in a hybridization oven (Turbo Speed hybridization oven, Bio/Can Scientific). The filters were then probed overnight at 42°C with <sup>32</sup>P-labeled probe (Oligolabeling  
30 kit, Pharmacia) corresponding to bases 334-2037 of BH10 clone. Ratner et al. (1985). The membranes were washed after hybridization and subsequently exposed (Kodak X-OMAT AR). The results obtained are presented in Figure 9. Figure 9A depicts the inhibition of HIV-1 transcript  
35 after 4h pretreatment with the construct prior to

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isolation of total RNA with GAPDH as loading control. Figure 9B depicts the results obtained after 24 h pretreatment prior to isolation of total RNA with 28S as loading control. The controls were (i) the inverse complement of the antisense; and (ii) the antisense construct with 2 point mutations.

#### Example 11

##### Antiviral Activity Assay

To test the antiviral activity of the antisense oligodeoxynucleotide phosphorothioate in acutely infected cells, HIV-IIIB laboratory strain of HIV-1 isolated from chronically infected H9 cells were used to infect P-PHA-stimulated CBMC at a viral titer of  $TCID_{50}=2000$  (viral stock =  $5 \times 10^3$   $TCID_{50}/ml$ ). After a two hour infection at 37°C, cells were washed free of unattached virus and resuspended in fresh medium containing 10 U/ml IL-2. Cells were plated at  $4 \times 10^5$  cell/well and the appropriate concentrations of insulin (Sigma Co.) or IGF-1 (Gibco Laboratories) as well as the concentrations of GP12A were added. The inhibition of HIV-1 activity in the cell culture supernatant was determined by the reverse transcriptase assay described in Example 11 after 4 days in culture. The results obtained are depicted in Figure 10.

#### Example 12

##### Reverse Transcriptase Assay

In order to determine the effect of GP12A on HIV reverse transcriptase (RT), the following experiment was performed.

Chronically infected H9/IIIB cells were seeded at  $4 \times 10^5$  cells/well with heat-inactivated serum and 1  $\mu M$  concentration of ODN/lipid formulation. The cells were washed with PBS after 24 hr preincubation with the ODN/lipid formulation and fresh medium containing 10%

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heat-inactivated serum plus ODN were added. The cells were held in culture for 5 days. The inhibition of HIV-1 activity in the cell culture supernatant was determined by RT assay on the culture supernatant. Statistical analysis by student's t-Test  $p < 0.02$  [unpaired].

RT assays were carried out by a modification of a previously published procedure. Boulerice et al. (1990) J. Virol. 64:1745-1744; and Lee et al. J. Clin. Microbiol. 25:1717-1722. Briefly, 50  $\mu$ l of clarified culture supernatant was added to 50  $\mu$ l of reaction cocktail containing 50 mM Tris HCl pH 7.9, 5 mM magnesium chloride, 150 mM potassium chloride, 0.5 mM ethylene glycol-bis( $\beta$ -aminoethyl ether)-N,N,N',N-tetraacetic acid, 0.5% Triton X-100, 2% ethylene glycol, 5 mM dithiothreitol, 0.3 mM reduced glutathione, 20  $\mu$ Ci titrated thymidine triphosphate and 50  $\mu$ g/ml of template primer [poly(rA).oligo(dT)] in polypropylene tubes. The tubes were agitated and then incubated at 30°C for 22 hours. The reaction was stopped by the addition of 1 ml of cold trichloroacetic acid (TCA). Newly synthesized DNA was precipitated on ice for at least two hours, and then collected on Whatman GF/C glass filters and rinsed two times with cold 10% TCA and absolute ethanol. Filters were dried for 20 minutes and counted for incorporated radioactivity. The results obtained are presented in Figure 11.

### Example 13

#### Toxicity of GPI2A

In order to determine the effect of GPI2A on the colony-forming ability and growth characteristics of normal cells (B4.14), the following experiments were performed. B4.14 cells were pretreated with 1  $\mu$ M oligomer for 3 days. The cells were subsequently washed with PBS, harvested and were then reseeded at a low density. The cells were then held in culture until

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colonies were formed (about 6-11 days) before they were stained. The results obtained are presented in Figure 12.

5 In order to determine the effect of GPI2A on growth of normal cells, the following experiment was performed. B4.14 cells were plated and subsequently grown in the presence of 1  $\mu$ M ODN for 3 days. The cells were reseeded into 100 mm tissue culture plates and allowed to grow in the presence of the ODN for the length of time indicated  
10 on the X-axis of Figure 13. Cells were then harvested and counted at the indicated times. The results obtained are presented in Figure 13. From Figures 12 and 13 it can be seen that GPI2A showed no toxicity in cell culture.

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Claims

1. At least one oligonucleotide comprising a nucleotide sequence sufficiently complementary to a region of human immunodeficiency virus (HIV) genetic material such that when bound to said region, the oligonucleotide substantially reduces expression of the HIV genetic material.
2. At least one oligonucleotide according to claim 1 wherein the region is selected from the group consisting of: between the 5' long terminal repeat and the first initiation codon of the gag gene; and the second splice acceptor site.
3. The at least one oligonucleotide according to claim 1 wherein the nucleotide sequence is complementary to the nucleic acid sequences selected from the group consisting of: +262 to +281; and +1189 to +1208.
4. The at least one oligonucleotide according to claim 1 wherein the nucleotide sequence is selected from the group consisting of:  
5' CCGCCCTCGCCTCTTGCCG 3'; and  
5' GGTCTTTTGGTCCTTGTCT 3'.
5. The at least one oligonucleotide according to claim 4 wherein the nucleotide contains at least one covalently bound sulfur atom.
6. The at least one oligonucleotide according to claim 5 wherein the nucleotide sequence is selected from the group consisting of:  
5' C'CG'CC'CC'TC'GC'CTC'TTG'CC'G 3'; and



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5' G'GTTTCTTTTG'GTCC'TTG'TCT 3';

wherein S stands for a sulfur atom.

7. A composition comprising a therapeutically effective amount of the at least one  
5 oligonucleotide according to claim 1 and a pharmaceutically acceptable carrier therefor.

8. The composition according to claim 7, further comprising a therapeutically effective amount of a lipid formulation.

10 9. The composition according to claim 8 wherein the lipid formulation contains a lipid mixture, liposomes or a cationic lipid preparation.

10. The composition according to claim 9 wherein the cationic lipid preparation is lipofectin.

15 11. A composition comprising a therapeutically effective amount of the at least one oligonucleotide according to claim 2 and a pharmaceutically acceptable carrier therefor.

20 12. The composition according to claim 11, further comprising a therapeutically effective amount of a lipid formulation.

13. The composition according to claim 12 wherein the lipid formulation contains a lipid mixture, liposomes or a cationic lipid formulation.

25 14. The composition according to claim 13 wherein the cationic lipid preparation is lipofectin.

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15. A composition comprising a therapeutically effective amount of the at least one oligonucleotide according to claim 3 and a pharmaceutically acceptable carrier therefor.

5           16. The composition according to claim 15, further comprising a therapeutically effective amount of a lipid formulation.

          17. The composition according to claim 16 wherein the lipid formulation contains a lipid mixture,  
10 liposomes or a cationic lipid preparation.

          18. The composition according to claim 17 wherein the cationic lipid preparation is lipofectin.

          19. A composition comprising a therapeutically effective amount of the at least one  
15 oligonucleotide according to claim 4 and a pharmaceutically acceptable carrier therefor.

          20. The composition according to claim 19, further comprising a therapeutically effective amount of a lipid formulation.

20           21. The composition according to claim 20 wherein the lipid formulation contains a lipid mixture, liposomes or a cationic lipid preparation.

          22. The composition according to claim 21 wherein the cationic lipid preparation is lipofectin.

25           23. A composition comprising a therapeutically effective amount of the at least one oligonucleotide according to claim 5 and a pharmaceutically acceptable carrier therefor.

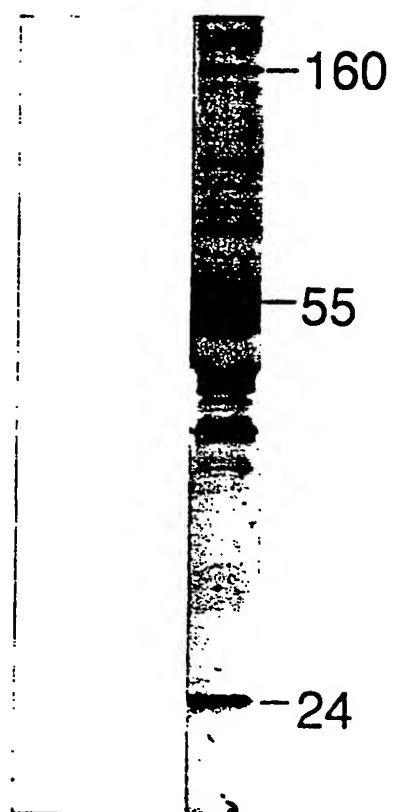
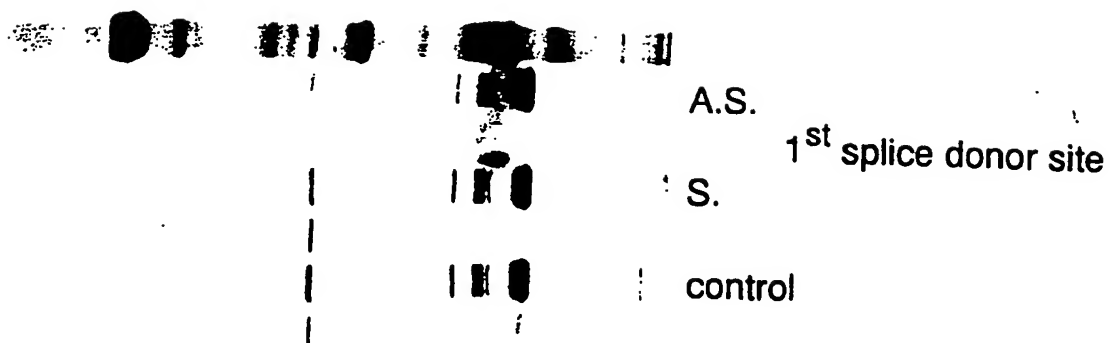
-33-

24. The composition according to claim 23, further comprising a therapeutically effective amount of a lipid formulation.

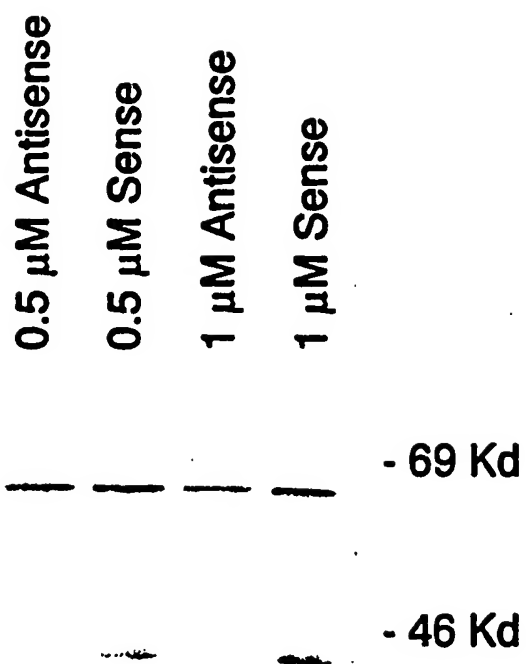
25. The composition according to claim 24  
5 wherein the lipid formulation contains a lipid mixture, liposomes or a cationic lipid preparation.

26. The composition according to claim 25 wherein the cationic lipid preparation is lipofectin.

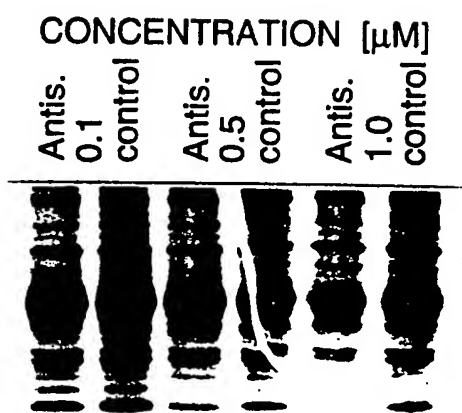
1/8

**FIGURE 1****FIGURE 2**

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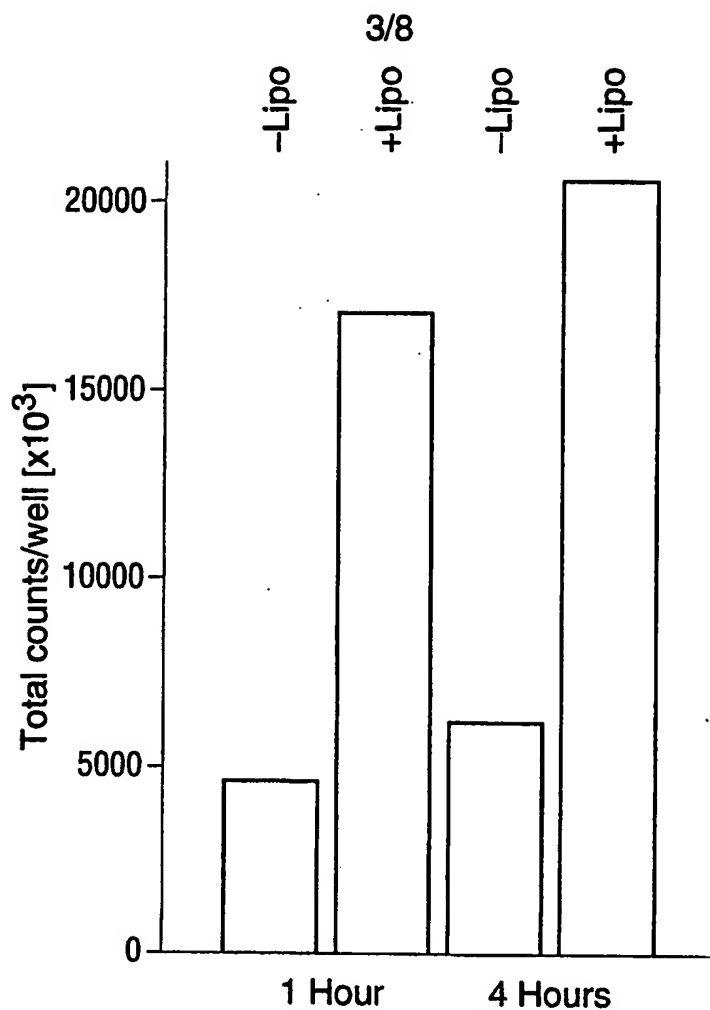
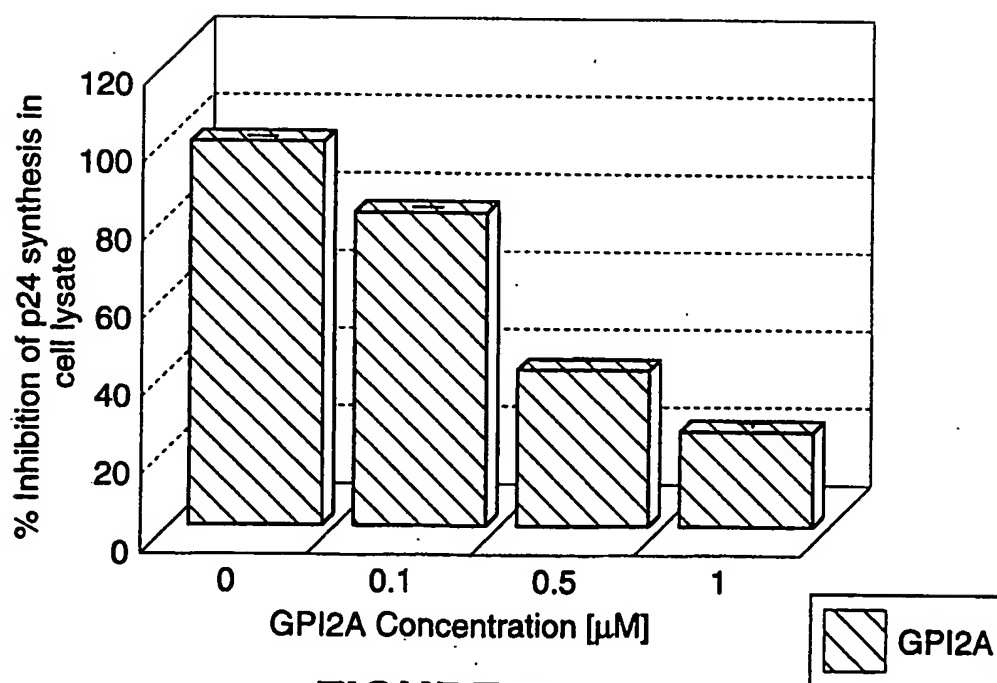


**FIGURE 3**

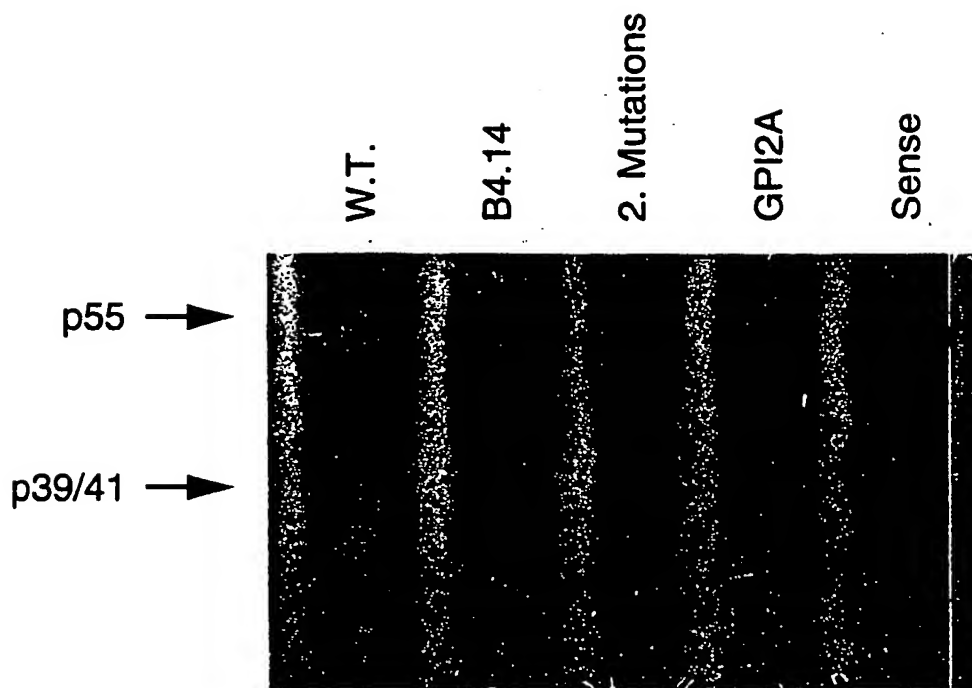


**FIGURE 4**

← p24

**FIGURE 5****FIGURE 8**

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**FIGURE 6**

**Antisense**

**Sense**

**2.Mutation**

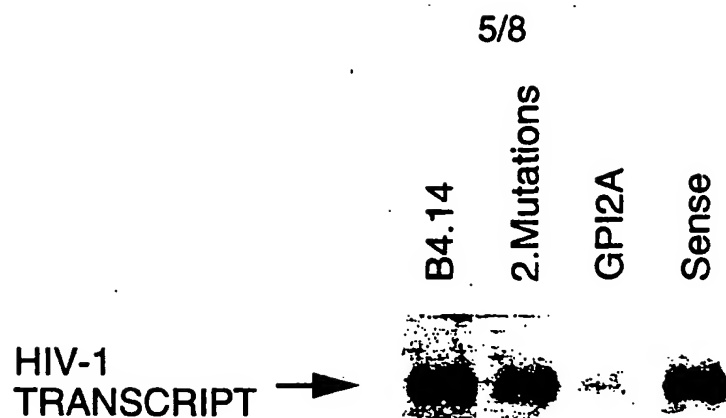
**B4.14**

**CMT3**

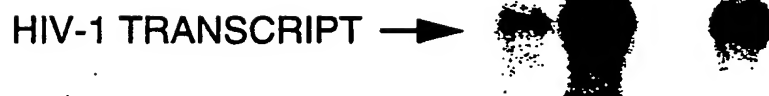
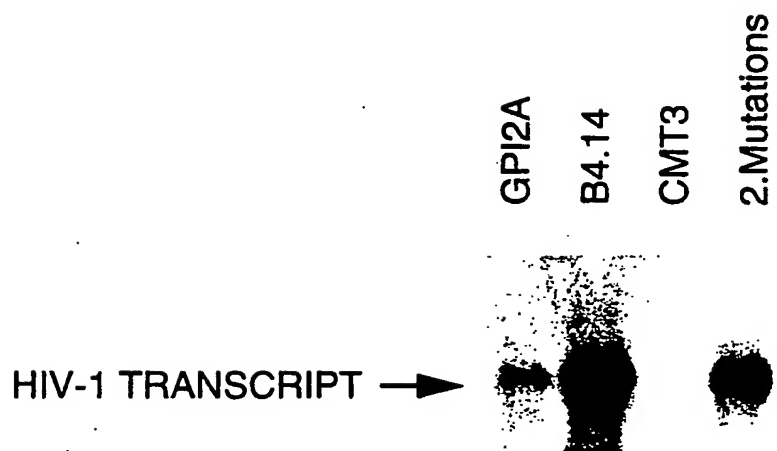
← **p24**

**FIGURE 7**

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**FIGURE 9A**

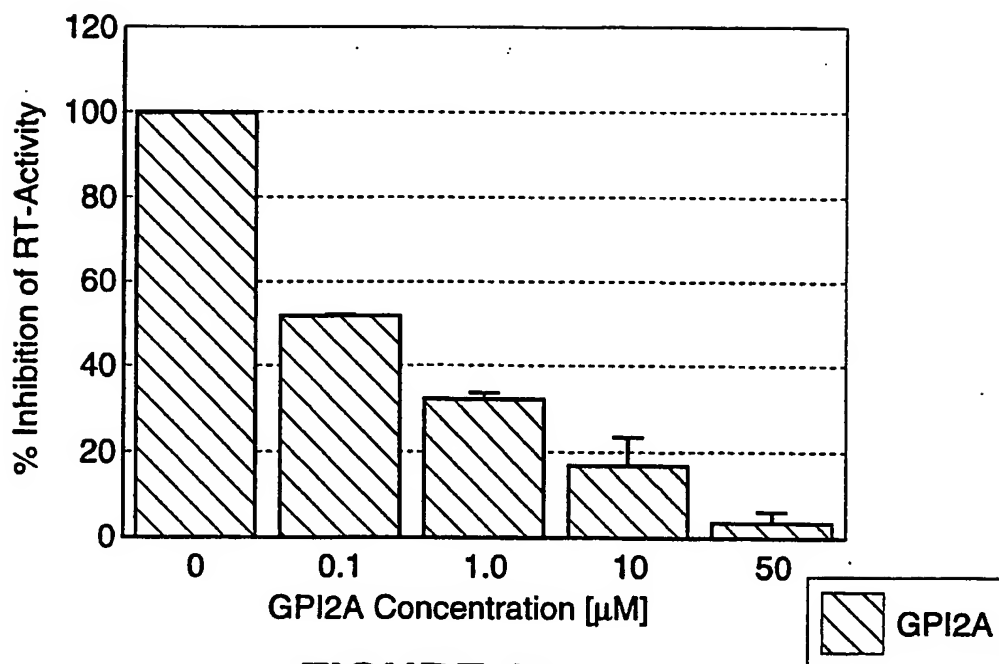
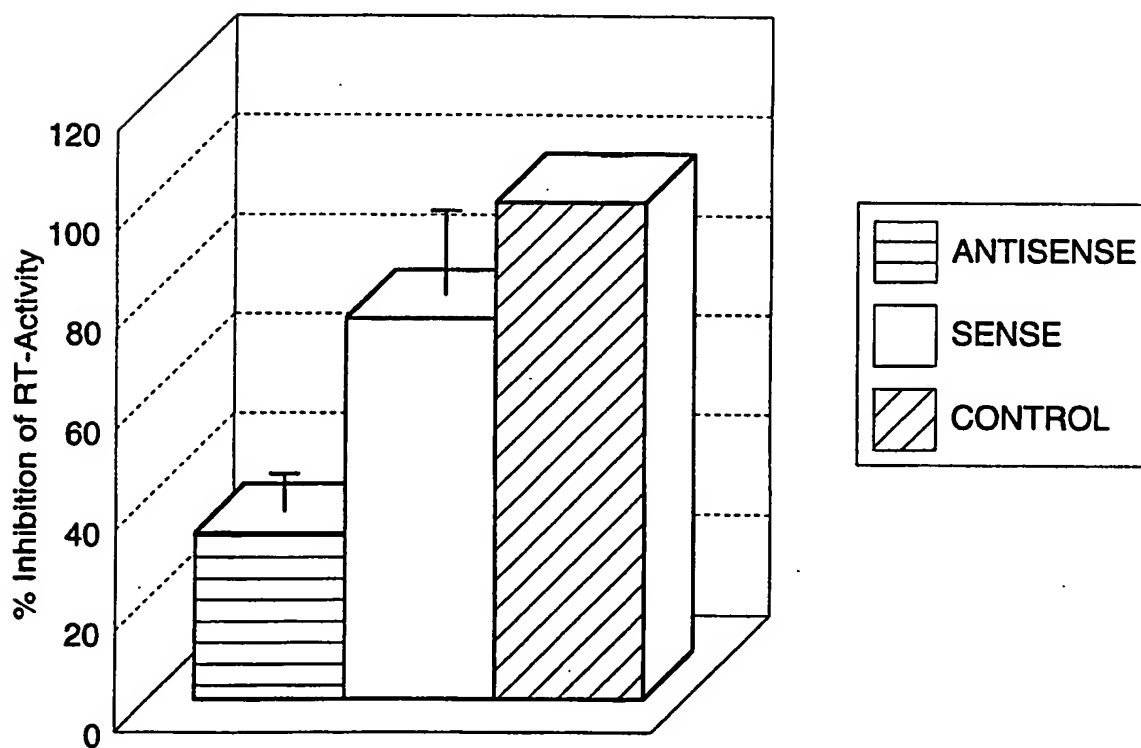


**FIGURE 9B**





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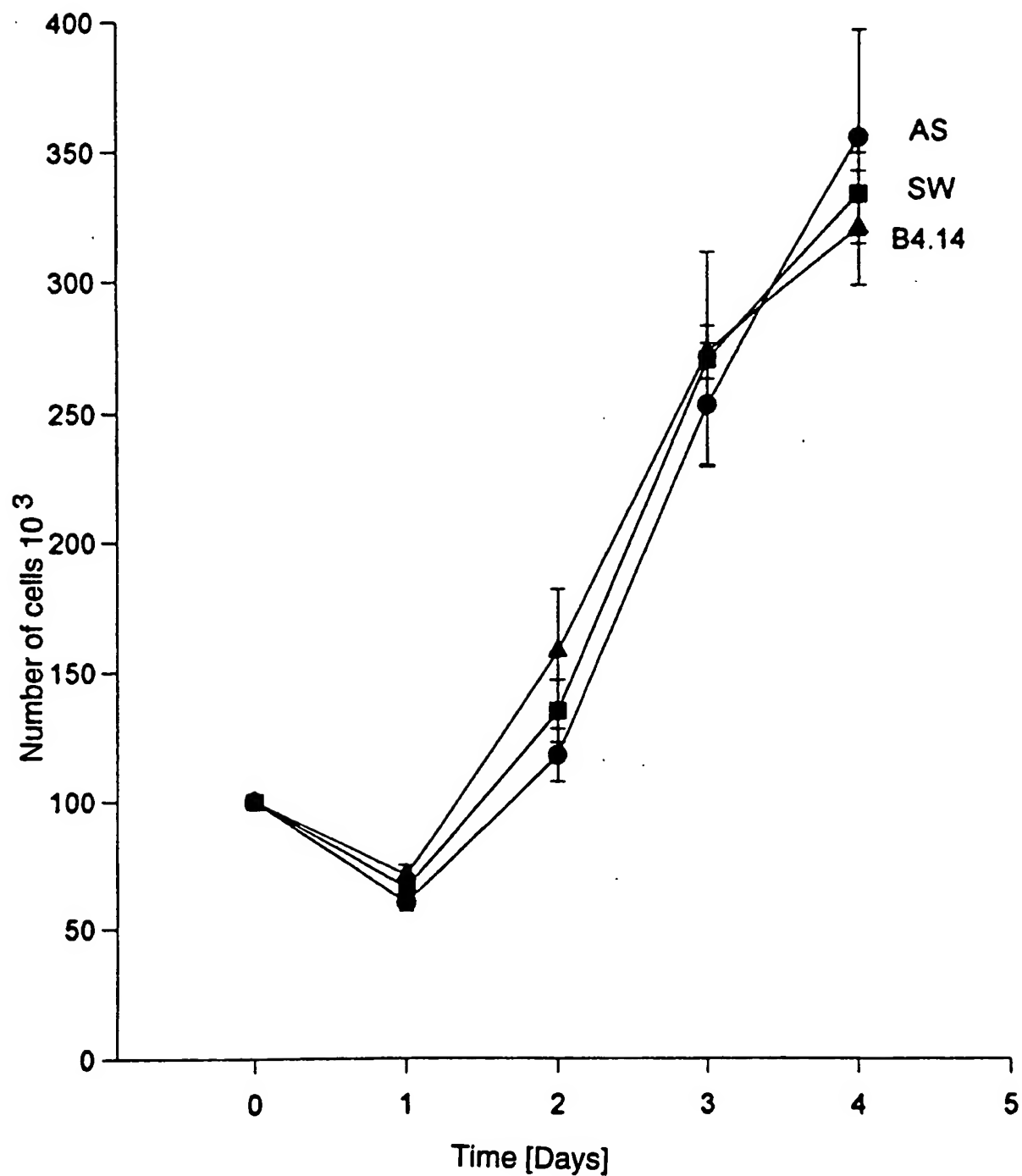
**FIGURE 10****FIGURE 11**

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**FIGURE 12**

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**FIGURE 13**

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